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Research paper

Irradiated mesenchymal stem cells improve the *ex vivo* expansion of hematopoietic progenitors by partly mimicking the bone marrow endosteal environment

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ABSTRACT

Mesenchymal Stem Cells (MSCs) regulate the growth and differentiation of Hematopoietic Progenitor cells (HPCs) through the release of soluble factors or through their differentiation into osteoblasts. We recently demonstrated that expansion of megakaryocyte (MK) progenitors *ex vivo* had reached a plateau when CD34$^+$ cells were grown with two optimized cytokine cocktails developed for the growth of MK. Hence, we sought to determine whether co-culture of CD34$^+$ cells with Bone Marrow (BM) MSCs could further increase the expansion of myeloid and MK progenitors. First, we tested the impact of cell–cell contact and pre-irradiation treatment of the MSCs to identify the condition that best supports HPC expansion. This screen revealed that HPC expansions were generally greater in the non-contact conditions, and that pre-irradiation of the MSCs appeared to be of added benefits. Improved expansion of both myeloid and MK progenitors in co-culture with irradiated MSCs without contact was subsequently confirmed. Next, cytokine array profiling was carried out to investigate why irradiation promoted progenitor expansion. This revealed that the levels of as many as 33 factors were potentially altered. ELISA confirmed the significant up regulation of NT-3 and IGFBP-2. Since, these factors are known to be released by and important for osteogenic and endothelial cells, we investigated and confirmed that irradiation of MSCs induced their rapid differentiation into osteogenic-like cells, but not into endothelial-like cells. Supporting this finding, expansions of myeloid and MK progenitors were increased when CD34$^+$ cells were co-culture with MSCs-derived osteoblasts. Altogether, these results indicate that the improved expansion of HPCs obtained with irradiated MSCs is due in part to their differentiation into osteoblast-like cells, thereby recreating an endosteal-like environment that provides improved support for HPCs expansion.

1. Introduction

All hematopoietic cells are derived from Hematopoietic Stem Cells (HSCs) which possess two key properties; *self-renewal* for maintenance or expansion, and *differentiation* to produce mature myeloid and lymphoid cells (Osawa et al., 1996). These processes are regulated by a combination of soluble and non-soluble factors present in the marrow microenvironment. It is now well established that HSCs and maturing hematopoietic cells are not randomly distributed in the marrow, as recent studies demonstrated that HSCs localized principally in close proximity to the endosteal surface in the so-called osteoblastic niche (endosteal zone),

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while differentiating Hematopoietic Progenitor Cells (HPCs) migrate to vascular niches (Kopp et al., 2005).

The conceptual framework of the osteoblastic and vascular niche scheme is that these niches provide appropriate stimuli to support the homeostasis of HSCs and the proper differentiation and maturation of committed HPCs, respectively. Each niche is thought to be composed of a complex mixture of extracellular proteins, soluble factors (cytokines and growth factors) and cellular components of both hematopoietic and non-hematopoietic origins. Several distinct cellular entities compose the non-hematopoietic portion of the marrow. These include osteoblasts, endothelial cells, adipocytes and fibroblast each with their own properties and specific roles, and in all likelihood all implicated in the regulation of hematopoiesis (Dennis and Charbord, 2002; Shiozawa et al., 2008).

A recent study done in mice has now demonstrated that bone marrow osteoblasts, chondrocytes, adipocytes and all of the colony-forming-unit fibroblastic cells are derived from rare resting Nestin$^-$ MSCs (Méndez-Ferrer et al., 2010). MSCs were previously recognized for their supportive roles on the growth and differentiation of HSCs and HPCs (Zhang et al., 2004; Wang et al., 2004). MSCs reside and can be derived from various tissues and organs, such as adult and fetal BM, Umbilical Cord Blood (UCB), spleen and amniotic fluid (Kode et al., 2009). Similarly to HSCs and Nestin$^+$ Mesenspheres, *in vitro* expanded MSCs have the capacity to undergo self-renewal and differentiate into a series of specialized cells, such osteoblasts (Jaiswal et al., 1997), chondrocytes (Johnstone et al., 1998), myocytes (Wakitani et al., 1995) and adipocytes (Pittenger et al., 1999). MSCs have also been shown to release of wide array of cytokines, chemokines and growth factors that can support hematopoiesis (Kode et al., 2009; Hwang et al., 2009) Co-cultures of HSCs and MSCs have confirmed that MSCs can support the expansion of HPCs *ex vivo* up to 4.6-fold by day-7 (Liu et al., 2010; Li et al., 2007a, 2007b), when grown in contact (Liu et al., 2010), and by 8.0-fold by day-14 (Robinson et al., 2006).

Osteoblasts have also important modulatory activities on hematopoietic cells both *in vitro* and *in vivo*. Human osteoblasts were first shown to secrete granulocyte-colony forming factor (G-CSF) and support granulopoiesis *ex vivo* (Taichman and Emerson, 1994). *In vivo*, osteoblasts regulate the pool of HSCs through Notch dependent pathways (Calvi et al., 2003). Consistent with this, a recent a study demonstrated that osteoblasts are more efficient than MSCs (or a mixture of both) for the expansion of hematopoietic repopulating and colony forming cells (Chitteti et al., 2010).

There is no consensus as to whether co-culture of hematopoietic cells with stromal cells should be done with or without contact, since both strategies offer distinct advantages. For instance, a study reported that human MSCs can support the formation of MKs and their full maturation when grown together in contact in the absence of exogenous cytokines (Cheng et al., 2000). Others have shown that direct contact between HSCs and BM stroma cells is not required for long term hematopoiesis by means of Transwell insert or encapsulation systems (Verfaillie, 1992; Xie et al., 2006; Liu et al., 2009). Conversely, most co-cultures of HSCs with osteoblasts reported to date have been done in contact condition (Taichman and Emerson, 1994; Chitteti et al., 2010).

Different methods have also been reported in the literature for the pre-treatment of MSCs prior to their use as feeder cells. Some investigators used irradiation treatment (Liu et al., 2010; Schattner et al., 1998; Gonçalves et al., 2006; Kawano et al., 2006; da Silva et al., 2005) or mitomicyn C (Flores-Guzmán et al., 2009) to limit MSC growth, while others have simply used untreated MSCs (Li et al., 2007a, 2007b; Robinson et al., 2006; Cheng et al., 2000; Wang et al., 2004). Ionizing radiations (γ, laser, X) of MSCs have been investigated and their effects on proliferation and differentiation of MSCs were recently reported (Schönmeyr et al., 2008; Greenberger and Epperly, 2009; Kushibiki and Awazu, 2009). However, little is known about the impact of irradiation on the capacity of MSCs to support HPC growth.

At this time, only a few studies have investigated the synergistic potential of MSCs with cytokine cocktails to improve expansion of MKs (Liu et al., 2010; Pallotta et al., 2009). In contrast to HSCs and HPCs, mature MKs localize exclusively in the vicinity of bone marrow vascular sinusoids, an environment shown to promote the formation and release of platelets even in the absence of thrombopoietin (TPO) (Avecilla et al., 2004). *Ex vivo* expansion of MK progenitors is currently under investigations as a mean to accelerate platelet recovery in the context of CB transplantation (Pineault et al., 2011; Mattia et al., 2008), since platelet recovery is significantly delayed with this rich HSC source (Rocha et al., 2004). Toward this, we recently optimized the cytokine cocktail OMPC (optimized megakaryocyte progenitor cocktail; SCF, TPO, FL) (Pineault et al., 2011) and BS1 (best setting 1; SCF, TPO, IL-6, IL-9) (Cortin et al., 2005) through the use of factorial (for cytokine selection) and central composite (to optimize the cytokine concentrations) designs for the differentiation of MK and expansion of MK progenitors, respectively. Unexpectedly, both cocktails induced equivalent level of MK progenitor expansions from Cord Blood (CB) CD34$^-$ cells, suggesting that expansion in serum-free medium supplemented with cytokines had likely reached a plateau (Pineault et al., 2011).

Hence, we sought to determine if co-culture of CB CD34$^-$ cells with MSCs might further increase the expansion of HPCs and MK progenitors based on the concept that MSCs have important implication in the regulation of HPCs. Unclear at this time however, is whether MSCs should be irradiated or not, and whether co-culture should be done with or without direct contact (Magin et al., 2009). In summary, our results indicate that improved expansions of HPCs including that of MK progenitors are best achieved with irradiated MSCs without direct cell contact. We also present a series of results that demonstrate that irradiation induces the differentiation of MSCs into osteoblast-like cells, and that this event is likely responsible for the improved expansion of HPCs achieved in co-culture.

2. Materials and methods

2.1. Isolation of CD34$^+$ hematopoietic UCB cells

Human UCB mononuclear cells and CD34$^-$ cell enrichment (Human Progenitor Enrichment Cocktail and StemSep column, StemCell Technologies, Vancouver, BC, Canada) were carried out as previously described (Pineault et al., 2011). Purity of

$CD34^-$-enriched cells was confirmed by flow cytometry (purity $75 \pm 5\%$).

2.2. Isolation and culture of MSCs

Human BM MSCs derived from healthy donors (between the ages of 45 and 75) were accepted as a gift from Dr. Jacque Galipeau (Lady Davis Institute for Medical Research, McGill University, Canada) or derived from whole BM cells (Lonza, Walkersville, MD, USA) as previously described (Çelebi and Elçin, 2009). MSCs were cultured in α-MEM, supplemented with 20% FBS (Invitrogen, Burlington, ON, Canada), 100 U/mL penicillin, and 1 ug/mL streptomycin at 37 °C in a humidified incubator (5% CO_2). Culture medium was renewed twice a week and cell viability was checked by trypan blue dye exclusion.

2.3. Osteogenic differentiation assay

BM MSCs were cultured in a humidified atmosphere of 5% CO_2 at 37 °C until confluence. Osteogenic differentiation was induced using DMEM (Invitrogen) medium supplemented with 10% FBS, 10^{-7} M dexamethasone, 0.2 mM ascorbic acid, and 10 mM glycerol 2-phosphate for 14 days (Çelebi et al., 2010). All chemicals were purchase from Sigma-Aldrich (St-Louis, MO, USA). After 14 days of cultures, the mineralization capacity was evaluated by Alizarin Red S (Sigma) staining.

2.4. Co-culture condition

$CD34^-$ cells were grown in serum-free medium (Pineault et al., 2008) supplemented with different combinations of human cytokines. 2×10^4 CB $CD34^+$ cells/mL were placed in cultures for the first 4 days with the cocktail OMPC (Pineault et al., 2011) (TPO 35 ng/mL, Stem Cell Factor (SCF) 10 ng/mL, Flt-3 ligand (FL) 11 ng/mL). Between day-4 and day-14, cell medium changes were carried out with the same medium but supplemented with the cytokine cocktail BS1 (TPO 30 ng/mL, SCF 1 ng/mL, IL-6 7.5 ng/mL and IL-9 13.5 ng/mL) optimized for the differentiation of MK cells (Cortin et al., 2005). All cytokines were purchased at Feldan Bio (Montréal, Canada) except for FL which was purchased at Peprotech (Rocky Hill, USA).

2.4.1. Co-culture condition tested

Irradiated (14 Gy) and non-irradiated BM-MSCs were first seeded in 24-well plates (2×10^4 cells/well) for 3 days. At day 0, MSCs were rinsed with PBS and UCB $CD34^-$ cells (2×10^4 cells/mL) were then plated onto the MSC layer or in transwell inserts on top of the MSCs (0.4 μm microporous filter, Corning Life Sciences, Lowell, MA, USA) in OMPC-medium. All cultures were diluted 1/2 with BS1-medium at days 4 and 10, except at day 6, when hematopoietic cell density was adjusted to 200,000 cells/mL. All cultures were done in duplicate and were maintained in a humidified atmosphere 5% CO_2 at 37 °C. Viable nucleated cells were counted manually with a hematocytometer with 0.4% trypan blue on days 4, 6, 10, and 14.

2.4.2. Co-culture of MSCs differentiated to osteoblasts with UCB $CD34^+$ cells

First, BM-MSCs were sub cultured in 24-well plates (2×10^4 cells/well) for 3 days then cultured up to 10 days in osteogenic medium. Then at day 0, osteoblast-derived cells were rinsed with PBS and UCB $CD34^-$ cells (2×10^4 cells/mL) were placed and cultured in transwell inserts (0.4 μm microporous filter).

2.5. Flow cytometry analysis

CD34-enriched CB cells grown for 6 and 14 days were phenotyped by flow cytometry using a FACS-Cy Flow ML (Partec, Görlitz Germany) as previously described (Cortin et al., 2005). At least 10,000 events were acquired for each sample, with dead cell and debris gated out by the forward and side-scatter and propidium iodide (PI, Sigma) profiles. The antibodies used were; anti-CD235a-conjugated to phycoerythrine (PE), anti-CD41a (GPIIb)-allophycocyanine (APC), anti-CD34 fluoresceine isothiocyanate (FITC), anti-CD45-APC, anti-CD73-PE, anti-CD105 FITC and corresponding control antibodies (mouse isotype APC, PE and FITC controls). All antibodies purchased at Becton Dickinson Pharmingen (Mississauga, Canada) except for anti-CD34 (Immunotech, Beckman Coulter, Marseille, France).

Alexa Fuor 488 Annexin V conjugates were used for apoptosis detection 24 h post irradiation following manufacturer's instruction (Invitrogen). A negative (cells in EGTA) and a positive control (cells incubated in 55 °C) were prepared to determine the negative and positive areas.

2.6. Progenitor assays

Assays for human clonogenic myeloid progenitor cells (Colony forming Cells (CFC)) were performed using the MethoCult SF H4436 according to the manufacturer's instructions (Stem Cell Technologies, Vancouver, Canada). In brief, 500 CB CD34-enriched cells or day-6 expanded cells and 600 day-14 expanded cells were seeded per mL of medium. MK progenitor assay (Colony Forming Unit-MK (CFU-MK)) was performed using the MegaCult-C system (Stem Cell Technologies) following manufacturer instructions. 3000 CB CD34-enriched cells or day-6 cells and 12,000 day-14 expanded cells were seeded per chamber. Colonies were scored based on their size, with mature MK-progenitor forming small colony (3–20 cells), and immature progenitor forming larger colony (≥ 50 cells). All cultures were done in duplicate and were maintained in a humidified atmosphere 5% CO_2 at 37 °C.

2.7. Antibody-based protein array system

Protein array RayBio Human Cytokine Array V kit (RayBiotech, Norcross, GA, USA) was done on day-10 cell-free supernatant taken from conditioned serum-free medium of irradiated or non-irradiated BM MSC (independent donors, $n = 3$) cultures according to the manufacturer's instructions. Densitometry analyses were done using the Gel Doc 2000 imaging apparatus and with ImageLab (Bio-Rad, Mississauga, ON, Canada). All values were normalized with the intensity of the positive and negative controls.

2.8. ELISA

I-309, NT-3 and IGFBP-2 in cell-free supernatant on 10-day were quantified using RayBio Human ELISA kit (RayBiotech) according to the manufacturer's instructions. Each sample was tested in duplicate for three different donors.

2.9. Immunochemistry of MSCs

Irradiated and non-irradiated BM MSCs were cultured in serum free medium for 6 and 10 days and then fixed with 3.7% formaldehyde (Sigma). Mineralization was evaluated by Alizarin Red S (Sigma) staining, osteopontin and VE-cadherin proteins were visualized by immunochemistry staining using an anti-osteopontin and anti-VE-cadherin (R&D Systems, Minneapolis, MN, USA) secretions by (R&D Systems) according to the manufacturer's instructions. For quantification, over 200 cells per condition were scored as positive or negative for each immunostaining.

2.10. Statistical analysis

The data were analyzed with SPSS 16.0 statistical software (SSPS Inc., Chicago, IL, USA). Statistical significance was evaluated based on one-way ANOVA analysis, Mann Whitney-*U* test and Student paired *t* test, value of $P<0.05$ was considered significant.

3. Results

3.1. Characteristic of the human MSCs

First, we confirmed the phenotype and morphology of the human BM MSCs from independent donors to be used in the context of co-culture by flow cytometry and osteogenic differentiation assay. Cytometry analysis confirmed that the human BM MSCs (passage 4–5) expressed the mesenchymal markers CD105 and CD73, but not the hematopoietic markers CD45 (Fig. S1A). Given the recent studies showing the important role of osteoblasts in the regulation of HSCs and HPCs growth and homeostasis (Taichman and Emerson, 1994; Chitteti et al., 2010), we also tested the capacity of these MSCs to undergo osteoblast differentiation when placed in osteogenic-differentiation. The latter was confirmed by the changes in morphology, from spindle shape to cuboidal, and by calcium deposit revealed by alizarin red staining (Fig. S1B).

3.2. Impact of contact and irradiation on the expansion of hematopoietic progenitor cells in co-culture with MSCs

The first objective of this work was to identify the best co-culture condition for the expansion of HPCs (*i.e.* myeloid and MK progenitors) from UCB CD34$^+$ cells. In all, four co-culture conditions schematized in Fig. 1 together with a control culture were tested. These were: 1) co-culture with MSC without irradiation with contact (WI C), 2) without irradiation without contact (WI WC), 3) with irradiation with contact (I C), 4) with irradiation without contact (I WC), 5) and the control condition in which UCB CD34$^-$ cells were grown in suspension without MSCs. All five conditions were tested in serum-free medium

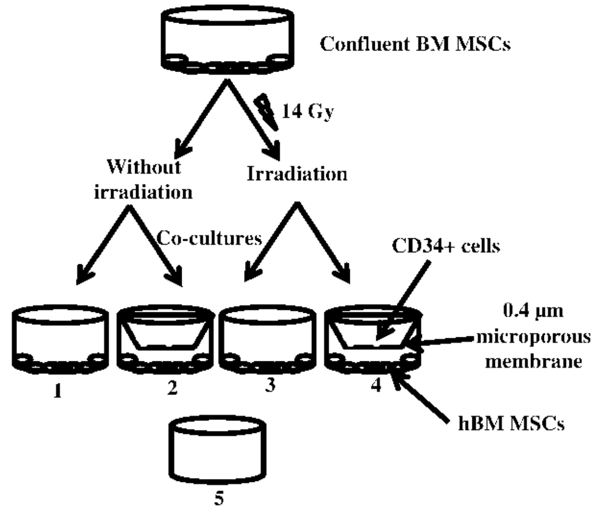

Fig. 1. Overview of the five culture conditions tested for the expansion of UCB CD34$^+$ cells. The impact of contact and irradiation (γ irradiation, 14 Gy) of MSCs on hematopoietic cell expansion and differentiation was assessed in four co-culture conditions; 1) without irradiation with contact (WI C), 2) without irradiation and without contact (WI WC), 3) with irradiation with contact (I C) and, 4) with irradiation and without contact (I WC). Growth without contact was done by culturing UCB CD34$^+$ cells in transwell inserts. The control condition (#5) consisted of UCB CD34$^+$-enriched cells cultured without MSCs. The same serum-free medium supplemented with exogenous cytokines was used for all conditions. The cytokine cocktails OMPC promoting MK progenitor expansion and BS1 promoting MK differentiation were used between day-0 and day-4, and day-4 to day-14, respectively.

supplemented with the cocktails OMPC (day 04) and BS1 (day 4–14).

In general, expansion of total nucleated cells (TNC) tended to be greater in most co-culture condition after 6 and 14 days of culture (Fig. 2A). The latter included a significant 2-fold increase in expansion at day-6 for the I WC condition compared to control (48.1 ± 11.9 *vs.* 24.2 ± 2.3). The expansion and differentiation kinetics of UCB CD34$^-$ cells in the four co-culture conditions are presented in the supplementary Fig. S2. Expansions of CD34$^-$ cells tended to be improved with most co-culture conditions (Fig. S2A–S2B). For instance, a 3.3- and 2.0-fold increase in the CD34$^+$ cell expansions were observed with the I WC condition compared to the control culture at day-6 and day-14, respectively. The I WC condition also tended to promote the expansion of CD34$^-$41$^-$ cells (1.7- and 1.6-fold) and CD235$^+$ cells (2.1- and 1.6-fold) compared to control after 6 and 14 days of culture (Fig. S2A–S2B).

To directly address the impacts of the different co-culture conditions on HPC expansions, we measured the total expansion of myeloid and MK (CFU-MK) progenitors by colony assays. Among the four co-culture conditions tested, expansion of myeloid progenitors tended to be greater in the two co-culture conditions without contact (Fig. 2B). Notably, myeloid progenitors outputs were increased by 12.7- and 2.4-fold compared to control when CD34$^+$ cells were co-cultured with irradiated BM MSCs without contact (I WC) at day-6 ($P<0.05$) and day-14 ($P>0.05$), respectively (Fig. 2B). In regards to the MK progenitors, increased expansion at day-6 was only observed in the co-culture conditions without contact (Fig. 2C), and only the I WC condition appears to have a positive effect at day 14. Taken together, these results suggest

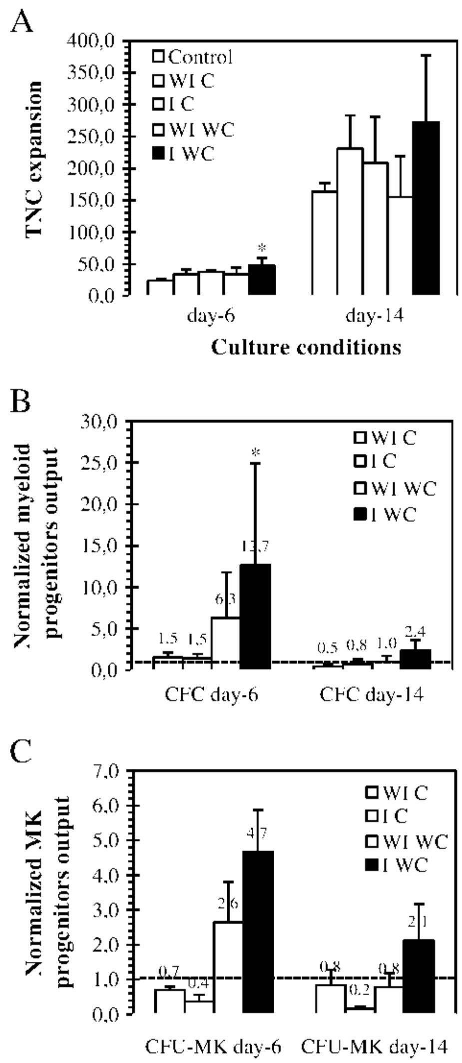

Fig. 2. Impact of contact and/or irradiation of BM MSCs on the expansion and differentiation of UCB $CD34^+$ cells in co-cultures. A) Cumulative Total Nucleated Cell (TNC) expansions per seeded cells. B) Total myeloid progenitor (CFU-GM, CFU-G, CFU-M, BFU-E and CFU-GEMM) outputs. The progenitor outputs were derived by multiplying the frequency of progenitors by the cumulative TNC expansion. C) Total MK progenitor (CFU-MK) outputs. Normalization to the control condition for each response was done at day-6 and -14 within each experiment, by dividing the test response by that of the control. The control condition was set arbitrarily at 1. Mean ± SEM of three independent experiments shown. * indicates significant differences ($P<0.05$).

that co-culture can be of added benefit for the *ex vivo* expansion of HPCs, and that the I WC condition appeared the most advantageous among the 4 co-culture conditions tested.

3.3. Ex vivo expansions of myeloid and MK progenitors are significantly increased in co-culture with irradiated MSC without contact

Consistent with the use of different human samples (UCB $CD34^+$ cell and BM MSCs preparations), large inter-experimental variations were observed for most biological responses presented above. To better characterize the differences between the I WC condition and control, we performed 10 additional independent experiments to evaluate the expansion and differentiation kinetics of UCB $CD34^-$ cells. No significant differences in overall cell expansion were observed at days 6 and 14 (Fig. 3A). However, the frequency of $CD34^+CD41^+$ cells was significantly (2.1 ± 0.7 *vs.* 1.3 ± 0.4, $P<0.05$) increased at day-6 (Fig. 3B), while that of $CD41^+$ cells was significantly reduced ($P<0.01$) at day-14 (Fig. 3C). However, no significant difference in the net production of $CD41^-$-MKs was observed ($P=0.38$ at day-14, data not shown).

In regard to myeloid progenitor expansions, the additional tests confirmed the increased expansion at day-14 (77.5 ± 27.5 *vs.* 44.3 ± 12.8, $P<0.05$) in the I WC condition, while the difference at day-6 was not significant (Fig. 3D). Based on the increased frequency of $CD34^+CD41^+$ cells obtained at day-6 in the I WC co-cultures, we expected a rise in MK progenitor expansion, since this phenotype corresponds in part to that of MK progenitors (Debili et al., 1992). Increased MK progenitor expansions were indeed achieved at day-6 (99.3 ± 25.8 *vs.* 55.0 ± 18.8, $P<0.05$) and also at day-14 (391.5 ± 172.0 *vs.* 248.9 ± 130.6, $P<0.05$) (Fig. 3E). These results confirm the positive impact of co-culture with MSCs (I WC condition) on the expansion of myeloid and MK progenitors from UCB $CD34^+$ cells.

3.4. Impact of irradiation on the secretion profile MSCs

The growth and differentiation kinetics (Fig. S2) of UCB $CD34^-$ cells as well as the expansion of myeloid and MK progenitors (Fig. 2) obtained in non-contact co-culture with BM MSCs often varied when the MSCs were irradiated. This suggests that the irradiation treatment may influence the secretion profile of the MSCs. To investigate this possibility, conditioned medium from non-irradiated or irradiated BM MSCs derived from three independent donors were analyzed for cytokine and chemokine secretions using a membrane-based cytokine array that detects 80 different growth factors and cytokines. Irradiated and non-irradiated BM MSCs secreted a vast array of factors, since a total of 71 and 75 cytokines and growth factors were readily detected, respectively (data not shown). The most secreted cytokines by irradiated MSCs were GRO, IL-6, IL-8, TIMP-1, and TIMP-2, while GRO, IL-6, IL-8, TIMP-1, TIMP-2, ONCOSTATIN, RANTES and MCP-1 were the most secreted by non-irradiated MSCs (Fig. 4). A total of 7 factors were found to be secreted at different levels between irradiated and non-irradiated BM MSCs ($P<0.05$, Fig. 4). Also, 26 additional factors showed some level variations though the differences failed to be statistically significant (Fig. 4).

Next, ELISA was used to confirm the potential up regulation of three of the seven growth factors identified as being present at different levels (NT-3, I-309 and IGFBP-2). This was conducted on new conditioned media derived from the same donors used for the cytokine array analyses. The increase in secretion levels for IGFBP-2 (4-fold) and NT-3 (2-fold) from irradiated BM MSCs was confirmed ($P<0.05$), while that of I-309 remained below statistical significance ($P=0.3$) (Fig. 5). Interestingly, both IGFBP-2 and NT-3 have been reported to stimulate osteoblast cell expansion and differentiation (Kveiborg et al., 2001; Togari, 2002).

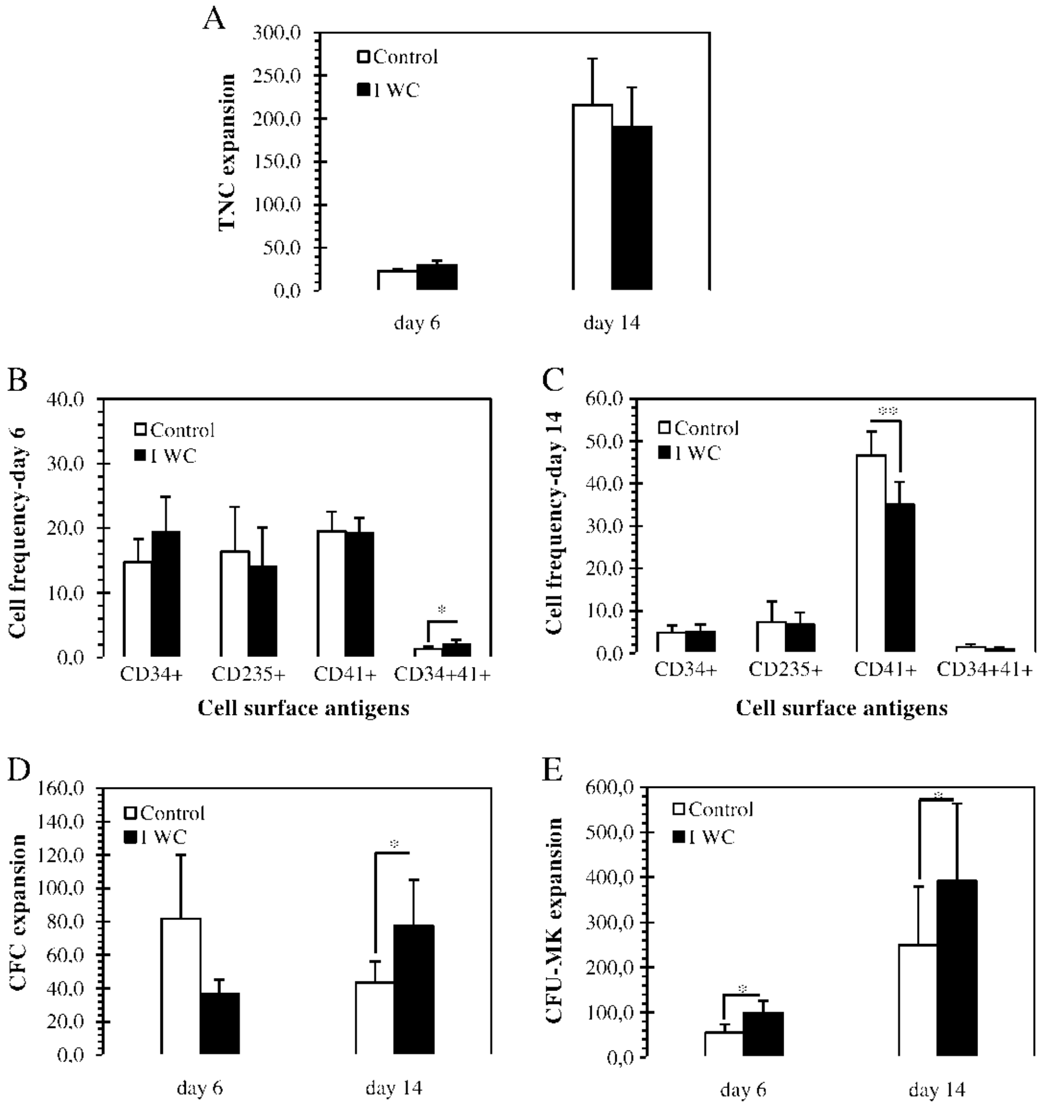

Fig. 3. Expansion of myeloid and MK progenitors can be improved by co-culture of UCB CD34^{-} cells with irradiated BM MSCs without contact. A) Cumulative TNC expansion per seeded cells at day-6 and day-14. B–C) Phenotypic analyses of the cells in cultures at days 6 and 14, respectively. D) Net expansion of myeloid (CFU-GM, CFU-G, CFU-M, BFU-E and CFU-GEMM) progenitors in culture at day-6 and day-14. E) Net expansion of MK progenitors (CFU-MK) obtained at day-6 and day-14 of culture. Net expansion of progenitors was calculated by dividing the progenitor output (see Fig. 2 legend) by the frequency of progenitors at day-0. Mean ± SEM of ten independent experiments shown. * indicates significant differences ($P<0.05$) and **($P<0.01$).

3.5. Pre-irradiation treatment of BM MSCs leads to osteogenic differentiation

The observed large divergence in the secretion profile of MSCs following irradiation suggests that such treatment may induce significant changes in the nature of the MSCs present in the co-culture. HSCs in the marrow reside in the osteoblastic niches (Zhang et al., 2003; Visnjic et al., 2004) and whole body irradiation in mice has recently been shown to lead to the expansion of ostoblasts, which can then support hematopoietic recovery from the surviving HSCs (Dominici et al., 2009). Hence, given the well established potential of osteoblast to sustain hematopoiesis, we investigated whether irradiation could induce osteoblast like differentiation of the MSCs. Alizarin red staining of calcium deposit and osteopontin immunochemistry were used as specific markers to investigate osteoblasts like differentiation (Post et al., 2008; Kim et al., 2005). Similarly to osteoblasts derived from BM MSCs cultured in osteogenic-differentiation medium, irradiated BM MSCs stained positively for both osteogenic markers 6 and 10 days post irradiation (Fig. 6A). In contrast, non-irradiated BM MSCs failed to produce calcium deposit or osteopontin and remained undifferentiated (Fig. 6A). Quantitative analysis of over 200 cells per condition of the osteopontin immunostaining revealed that essentially none and all of the MSCs and irradiated-MSCs stained positive for this osteogenic marker, respectively. Calcium deposit could also be the results of large scale induction of apoptosis, however, this scenario appears unlikely since the proportion of apoptotic cells in MSCs 24 h post-irradiation was only increased from 7.0 ± 5.0% to 13.4 ± 3.7% ($n = 2$), and

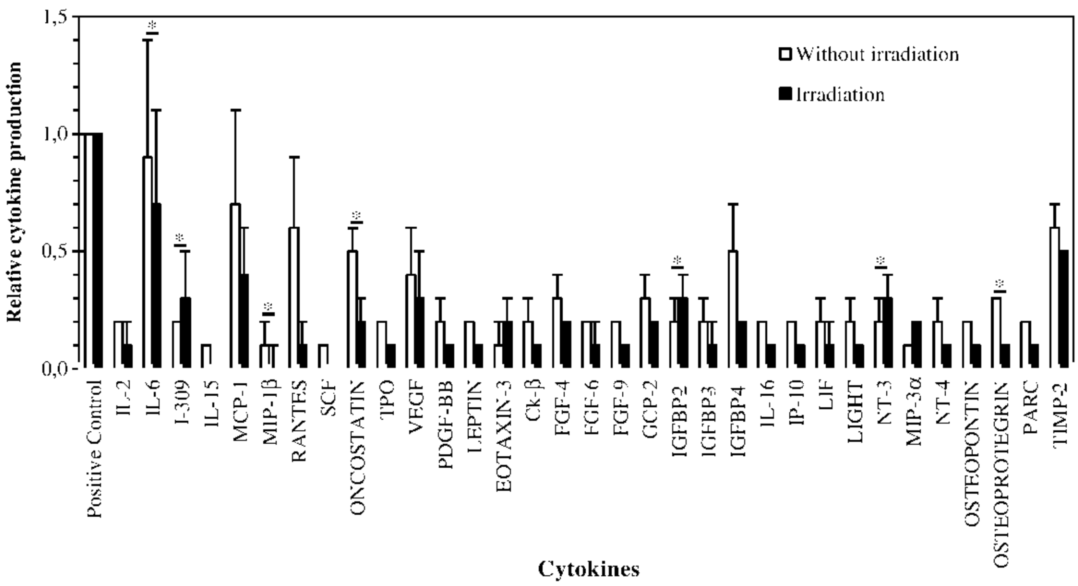

Fig. 4. Impact of irradiation on the secretion of soluble factors from BM MSCs. The expression level of a total of 33 soluble factors (cytokines or growth factors) tended to be affected between non-irradiated and irradiated BM MSCs (mean ± SEM of three independent experiments shown). For each condition, all cytokine signals were normalized with that of the positive and negative controls; (mean of cytokine intensities minus mean of negative control intensities) divided by positive control intensities. * indicates significant differences ($P<0.05$).

the proportion of viable cells remained high (69.0 ± 3.8% *vs.* 78.2 ± 7.0, Fig. S3).

To determine whether irradiation could also lead to endothelial differentiation given the presence of I-309, irradiated and non-irradiated MSCs were also stained for the endothelial specific adhesion molecule VE-cadherin (Vestweber, 2008). None of the 200 analyzed MSCs and irradiated MSCs expressed detectable level of extracellular VE-cadherin at both day 6 and day 10 post irradiation (Fig. 6B), while it was readily detected in all of the Human Umbilical Vein Endothelial Cells (HUVEC). Finally, we did not investigate whether irradiation of the MSCs induced adipocyte differentiation based on a previous report demonstrating that irradiation inhibited adipogenesis of MSCs (Yang et al., 2010). Taken together, these results demonstrate that irradiation leads to the spontaneous differentiation of BM MSCs into osteoblast-like cells.

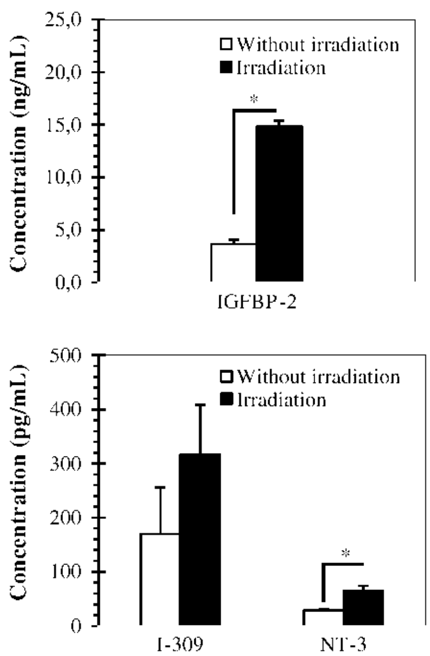

Fig. 5. Confirmation of the impact of irradiation on the secretion levels of IGFBP-2, I-309 and NT-3 from BM MSCs. The levels of each protein in conditioned media of irradiated and non-irradiated MSCs at day-10 were measured by ELISA. Mean ± SEM of three independent experiments shown. * indicates significant differences ($P<0.05$).

3.6. Osteoblasts-derived from MSCs support the expansion of myeloid and MK progenitors

According to the results presented above, the increase in myeloid and MK progenitor expansion obtained in co-culture with irradiated MSCs without contact could be the results of their differentiation into osteogenic-like cells after the irradiation, leading to differences in the secretion of soluble factors. To directly test this possibility, osteoblasts were first derived from MSCs from four independent donors induced to differentiate into osteoblast for 7 days with osteogenic differentiation medium. Then, UCB $CD34^+$ cells were co-cultured without contact with these osteoblasts (non-irradiated) to determine their capacity to support myeloid and MK progenitor's expansions. As shown in Fig. 7A, co-culture with osteoblasts significantly increased myeloid progenitor expansions by 1.7 (19.1 ± 3.3 *vs.* 11.7 ± 4.6) and 1.5-fold (155.0 ± 63.6 *vs.* 98.1 ± 66.7) at days 6 and 14 *vs.* control ($P<0.05$.). MK progenitor expansions were also significantly increased by 3.2 (31.4 ± 5.5 *vs.* 9.8 ± 5.7) and 2.2-fold (255.4 ± 141.1 *vs.* 127.3 ± 86.2) at day-6 and 14, respectively (Fig. 7B, $P<0.01$). Taken together,

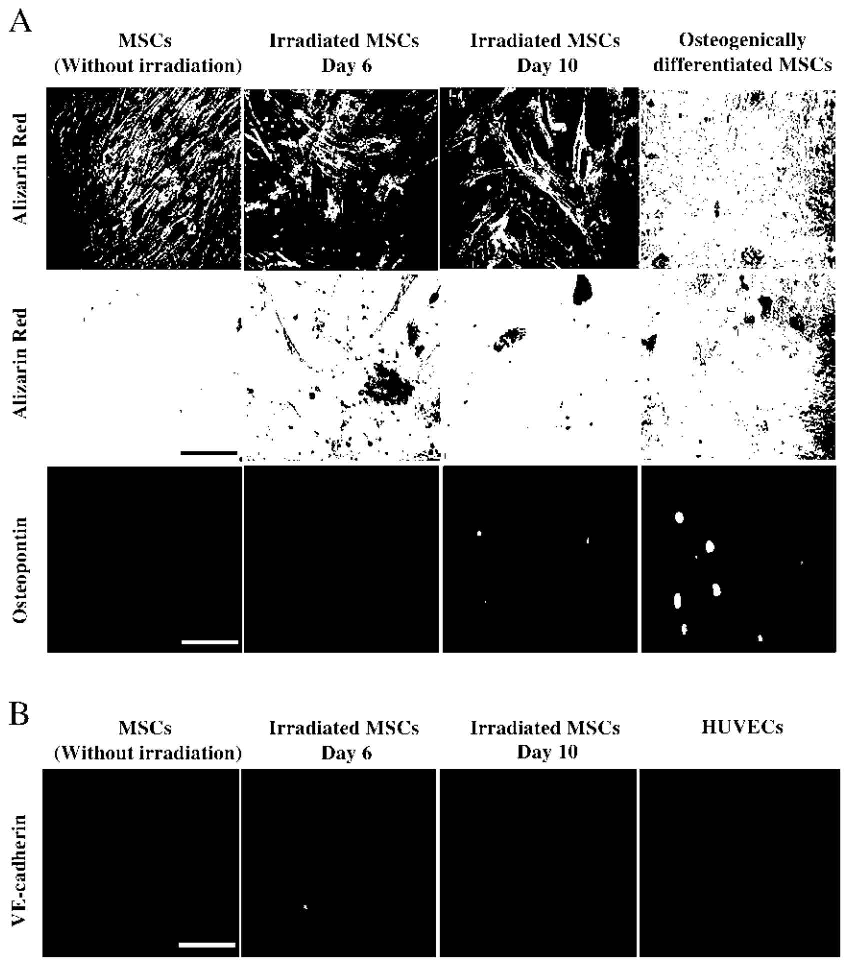

Fig. 6. Production of osteoblasts-like cells following pre-irradiation treatment of BM MSCs. A) In contrast to non irradiated MSCs, irradiated MSCs formed calcium deposits (alizarin red staining) and stained positive for Osteopontin 6 and 10 days post-irradiation. Osteoblasts derived from differentiated MSCs are shown as positive control. B) VE-cadherin staining of non-irradiated and irradiated MSCs 6 and 14 days post-irradiation. HUVEC were used as positive control. Phase contrast microscopy, bars = 100 μm.

these results support the previous results suggesting osteoblast-like differentiation induced by pre-irradiation treatment of MSCs is largely responsible for the increased expansion of HPCs and MK progenitors in co-culture of HSCs with MSCs.

4. Discussion

The present study had for principal objective to determine whether co-culture of UCB CD34$^-$ cells with MSCs could improve the expansion of MK progenitors which had reached a plateau in serum-free culture supplemented with cytokines. The principal interest in increasing the expansion of MK progenitors *ex vivo* is that co-transplantation of these cells with unexpected HSCs could help reducing the periods of thrombocytopenia associated with CB HSC transplantation. Improved expansion of this rare population was confirmed in co-culture with irradiated BM MSCs without contact. This study also demonstrated that the marked differences in the growth promoting potential between irradiated and non-irradiated MSCs originate from the differentiation of irradiated MSCs into cells with osteogenic properties, refereed herein as osteoblast-like cells. Co-culture of CD34$^+$ cells with osteoblasts derived from MSCs confirmed that this lineage posses the intrinsic potential to support the *ex vivo* expansion of both myeloid and MK progenitors.

Many studies using MSC as feeder cells for HSCs have used irradiated MSCs. However, the impact of irradiation on the growth promoting activity of MSCs on hematopoietic cells and on their cytokine production had not yet been reported in the literature. Irradiation of human BM MSCs was previously shown to inhibit in a dose-dependent manner their proliferation up to two weeks post-irradiation but thereafter, the residual surviving cells regained their normal proliferation rate (Li et al., 2007a, 2007b), which highlights their radio resistant characteristics. Following clues provided by the secretion profiling experiments, we discovered that irradiated MSCs had acquired properties (calcium deposits and osteopontin secretion) typically associated with osteoblasts. This finding is certainly of high relevance since osteoblasts are known regulators of HSCs and HPCs (Calvi et al., 2003). Osteoblast differentiation of MSCs following irradiation is further supported by other reports. Indeed, laser irradiation promotes the

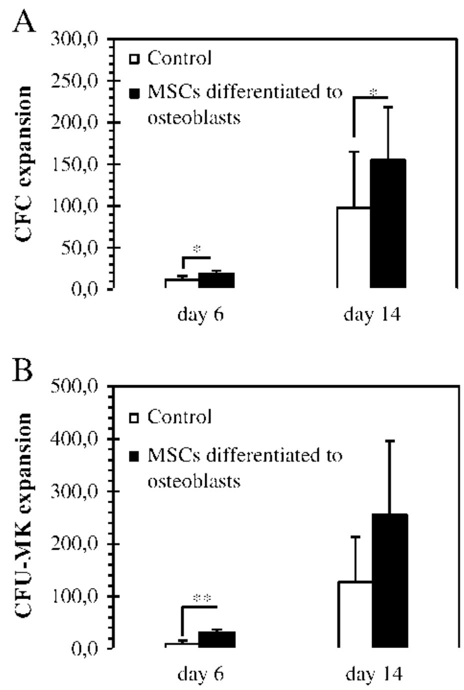

Fig. 7. Co-culture of UCB CD34$^+$ cells with osteoblasts derived from differentiated BM MSCs increases the expansion of myeloid and MK progenitors. A) Net expansion of myeloid clonogenic progenitors obtained after 6 and 14 days of culture. B) Net expansion of MK progenitors (CFU-MK) obtained at day-6 and day-14 of culture. Co-cultures were done with non-irradiated osteoblasts without contact. Mean ± SEM of four independent experiments shown. * indicates significant differences $P<0.05$ and $^{**}P<0.01$.

extracellular calcification of MSCs (Kushibiki and Awazu, 2009) and enhances BMP-induced osteoblast differentiation by stimulating the BMP/Smad signaling pathway (Hirata et al., 2010). In addition, electromagnetic fields (gamma irradiation) promote osteogenic differentiation of MSCs and concurrently inhibit adipocyte formation (Yang et al., 2010).

The improved expansion of HPCs achieved in the absence of direct cell contact supports the concept that secreted growth factors mediate the bulk of the MSC proliferative stimuli. For instance, MSCs have been shown to secrete a wide array of cytokine including early acting cytokines known to maintain HSC in quiescence or promote their self-renewal or differentiation. These include SCF, TGF-β, Flt-3, LIF, OSM, BMP-4, IL-1, IL-6, IL-7, IL-8, IL-11, IL-12, IL-14, and IL-15 (Hwang et al., 2009; Schinköthe et al., 2008). In absence of contact, HPC and MK progenitor expansions were also improved with irradiated MSCs suggesting that perhaps their profile of cytokine secretion was modified by the irradiation treatment. It is now clear that the divergence in cytokine profile between irradiated and non-irradiated MSCs is likely due to the differentiation of MSCs into osteoblast-like cells. Of the 33 cytokines identified as potentially modulated, seven factors showed significant differences including that of I-309, IGFBP-2 and NT-3. The increased levels of two of three of these factors were confirmed by ELISA (IGFBP-2 and NT-3). These factors were selected because of their potential implication in the maintenance of osteoblasts, endothelial cells and HPCs (Huynh et al., 2008). Conversely, four other factors were found to be reduced significantly in irradiated MSCs-conditioned medium (IL-6, MIP-1β, oncostatin and osteoprotegrin), and 24 others appeared down regulated though the differences were not significant.

The 4-fold increase in IGFBP-2 levels is of significant interest considering recent reports demonstrating its potential to expand HSCs and HPCs when used with other cytokines (Huynh et al., 2008; Zumkeller and Burdach, 1999). IGFBPs are a family of six circulating proteins that binds to Insulin Growth Factor-1 (IGF-1) and IGF-2, which modulates the biological effects of the IGFs (Corbo et al., 2010). IGFBP-2 mRNA is expressed in trabecular and cortical osteoblasts, some trabecular and subendocortical osteocytes, intracortical endothelial cells of blood vessels, and periosteum (Minuto et al., 2005). On the other hand, a 2-fold increase in NT-3 levels was also found in the supernatant of irradiated MSCs. The potential role of NT-3 in the regulation of hematopoiesis is at this time undefined. Interestingly, adult and cord blood NT-3 levels have been reported to correlate positively with that of FL, an important growth factor for HSCs and HPCs. NT-3 is a growth factor which has activity (survival and differentiation) on certain neurons of the peripheral and central nervous system (Malamitsi-Puchner et al., 2005). Neural regulation of bone metabolism mediated by osteoblastic and osteoclastic cells has been demonstrated by Togari (2002). It was also confirmed that osteoblasts can produce the neurotrophic factors NGF and BDNF (Mogi et al., 2000), and that NGF can act as a colony stimulating factor, as it stimulates bone marrow precursor cells to proliferate (Chevalier et al., 1994).

We focused our efforts in this work on factors that were upregulated. However, it is likely that some of the factors found at reduced levels may play a role in the growth promoting effects of irradiated MSCs. Certainly, future work will be required to determine whether NT-3, IGFBP-2 or other down regulated or up regulated soluble factors identified herein play or not an important role in the growth promoting potential of the osteoblast-like cells on HPCs. In conclusion, this study demonstrates that co-culture of UCB CD34$^-$ cells with irradiated BM MSCs or MSC-derived osteoblasts without contact is of added benefit for the expansion of HPCs and MK progenitors when used with cytokine cocktails promoting MK expansion. These findings provide additional support for the incorporation of MSCs in culture protocols designed for the expansion of HPC. Moreover, since the growth promoting potential of MSCs on progenitors is independent of cell-cell contact, the use of MSC-conditioned media free of serum represents an attractive avenue to incorporate the growth promoting activity of these cells in culture expansion protocol already approved for clinical trials.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at doi:10.1016/j.jim.2011.06.006.

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